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Discrepant microbiome configurations between hereditary nonpolyposis and sporadic colorectal cancer

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ABSTRACT

Objective: Hereditary nonpolyposis colorectal cancer (HNPCC) is a kind of well-known hereditary colorectal cancer (HCRC). This study aims to distinguish the gut microbiome between patients suffering from HNPCC and sporadic colorectal cancer (SCRC).

Methods: 10 HNPCC patients, 14 SCRC, and 14 normal controls were enrolled in this study. 16S rDNA V4 gene sequencing was performed on stool microbiome of these subjects using Illumina Miseq to analyze the fecal microbioata.

Results: The fecal microbiota configurations of patients with HNPCC and SCRC were significantly differed compared to controls ($p < 0.002$, $p = 0.031$, respectively), while the variation in SCRC was higher than that in HNPCC. In addition, fecal microbiota of patients with HNPCC and SCRC were significantly different ($p = 0.001$). The genus *Parvimonas* was significantly predominant in SCRC patients compared to HNPCC patients and a significant correlation was seen with *Fusobacterium*, *Brevibacillus, Methylobacterium, Granulicella,* and *Phenylobacterium*.

Conclusions: The significant difference in the gut microbiota between HCRC and SCRC highlights the underlying difference in microbial basis of these two phenotypically similar colorectal cancer types. *Parvimonas* with a higher abundance in SCRC patients relative to those with HCRC, may participate in SCRC pathogenesis and development.

Keywords: Colorectal cancer, gut microbiota, hereditary nonpolyposis colorectal cancer, *Parvimonas*

INTRODUCTION

Colorectal cancer (CRC) is a cancer with 3rd wide-spread and the 2nd cause of death due to cancer in the world. Recent studies estimated over 1,360,000 contemporary CRC cases and almost 700,000 global deaths annually[1,2]. CRC may occur resulting from a complex interaction between genetic, lifestyle, and environmental factors [3]. Increasing evidence supports the existence of a relationship between gut microbiota dysbiosis and CRC[4-8]. However, whether gut microbial dysbiosis participates in CRC or is just a consequence of CRC, is unknown. Hereditary nonpolyposis colorectal cancer (HNPCC), clinically most well-known hereditary colorectal cancer (HCRC), is caused by autosomal dominant heterozygous germline mutations[9-12]. If gut microbial dysbiosis occurs as a consequence of CRC, microbiota configurations of sporadic colorectal cancer (SCRC) should be similar to HCRC. Conversely, if gut microbiota of SCRC significantly differs from that of HCRC, it may be inferred that gut microbiota is a causal factor of CRC. Furthermore, among the specific microorganisms associated with CRC, some taxa that are more prevalent in patients with SCRC than in patients with HCRC, may be the contributing bacteria for CRC. In the present study, we aim to distinguish the gut microbiome between patients suffering from HNPCC and SCRC through performing 16S rDNA V4 gene sequencing on stool microbiome of these patients as well as healthy controls to profile gut microbiome alteration.

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METHODS

Patient recruitment, sample collection, and DNA preparation

A clinical group of 10 patients of HNPCC and 14 of SCRC, from the 7th medical center of People's Liberation Army General Hospital, were recruited. In addition, 14 healthy subjects were recruited. All

patients with CRC were examined and categorized by endoscopy and histology; the inclusion criteria for patients with HNPCC was based on the Amsterdam criteria II[13]. Sequencing analysis showed that patients with HNPCC were MMR gene mutation carriers while patients with SCRC did have MMR or APC gene mutation. All healthy participants voluntarily provided stool samples prior to colonoscopy and colonoscopy showed the absence of colorectal lesions.

Stool samples from individuals with CRC (HNPCC and SCRC) and healthy subjects prior to bowel preparation for colonoscopy were collected at home and immediately frozen at a low temperature (-20°C), or collected in hospitals and then frozen at - 80°C. According to the provider's instructions, DNA was extracted and analyzed by the Qiagen qiaamp DNA stool Kit (Qiagen).

16s rRNA Sequencing to profile microbiota

To profile microbiota combination, the hypervariable region (V4) of 16S rRNA gene was amplified using the bacterial universal primers for the 16S rRNA V4 region:

Forward primer: 5'-NNNNNNNNGTGCCAGCMGCCGCGGTAA

Reverse primer: 5'-NNNNNNNNGGACTACHVGGGTWTCTAAT

"NNNNNNNN" represents the 8-base unique barcode for each sample.

The PCRs were performed using primers and and a Taq DNA polymerase (New England biological laboratory). PCR conditions were: 95°C 2 min, followed by 25 cycles of 95°C 30 sec, 55°C 30 sec, 72°C 30 sec, and 72°C 7 min. The PCR amplification products were purified by rapid PCR Purification Kit (Qiagen) and quantified by hot nano drop nd-2000 spectrophotometer (nucliber). Equal concentrations of PCR products were then pooled together and sequenced using Illumina MiSeq platform.

Data analysis

Low-quality sequencing reads were filtered out which was implemented in QIIME by applying default settings and a minimum acceptable 20 points. Check primer and barcode sequence. After filtering, search (based on genetic similarity threshold ≥ 97%) clusters high-quality read data into operable classification units (OTUs). Then RDP algorithm and rdp-ii database were used to analyze OTU based on classification. Principal coordinate analysis (PCoA), principal component analysis (PCA), and Anosim analysis was preformed to compare the differences among bacterial communities. For correlations between the different microbiota of patients with SCRC and HNPCC, the correlation of Spearman was further assessed and the correlation graph was obtained using the corrplot package in R. LEfSe analyses using the Kruskal–Wallis rank sum test was applied to detect significant differences and Linear discriminant analysis (LDA) scores were generated to estimate the effects (threshold ≥ 4.0).

Validation of *Parvimonas* and *Fusobacterium* by qPCR

Tm, guanine-cytosine (GC) content and secondary structures (supplementary table S1) were assessed by qPCR using DBI Bestar SybrGeen qPCR mastermix.

Statistical analysis

SPSS20.0 and R (V3.2.3) were applied for analyzing data. Continuous variables were represented by median and classified variables were represented by numbers. Non-parametric test assessed continuous variables and chi-square test evaluated classified variables. In addition, Anosim analysis was used to test whether the difference between (two or more) groups was significantly greater than that within groups. Wilcoxon rank-sum test analyzed differences between samples in different groups. *P* value < 0.05 indicates a significance.

RESULTS

Patient characteristics

The hereditary pattern of a family with HNPCC was autosomal dominant inheritance and was shown in Figure 1. Among the 10 patients with HNPCC (hereafter referred to as patients with HCRC), 6 were man. Besides, the medium age of diagnosis was 45 years old (Table 1). Among the 14 patients with SCRC, 8 were male, and the median age of diagnosis was 49 years old (Table 2). Both age of diagnosis and gender distribution between patients with HCRC and SCRC were comparable without differences (p = 0.153, p = 0.611, respectively).

Distinct fecal microbiota configurations between SCRC and HCRC

The average alpha diversity differed significantly between SCRC and healthy controls (p = 0.011), whereas comparison of HCRC and healthy samples, HCRC and SCRC, revealed no significant differences (p = 0.189, p = 0.206) (Figure 2A, supplementary figure S1).

Using PCA, we assessed the dissimilarity in fecal microbiota communities among the patients with SCRC, HCRC, and healthy controls. Interestingly, the distance between SCRC and healthy controls were

far than that between HCRC and healthy controls (Figure 2B). Based on PCoA and Anosim analyses, the microbiota community in the samples obtained from SCRC patients was different from that of samples of HCRC patients (Anosim, $p = 0.001$; Figure 2C). Further, the microbiota structure dissimilarity between samples obtained from SCRC patients and controls was significantly higher than that between HCRC patients and controls (Anosim, $p < 0.002$, $p = 0.031$, respectively; Figure 2C). Collectively, the data suggests that SCRC microbiomes were more dysbiotic than HCRC microbiomes.

Comparison between the taxonomic alterations in SCRC and HCRC gut microbiota

To further analyze the difference in the microbiota community between patients with SCRC and those obtained from HCRC ($p = 0.001$), we performed DESeq analysis to identify the different taxa in these two groups. Higher abundances of *Firmicutes* and *Proteobacteria* were seen in control group than in SCRC and HCRC groups. In contrast, *Bacteroidetes, Actinobacteria*, *Fusobacteria,* and *Acidobacteria* were much more affluent samples obtained from people suffering from SCRC and HCRC, than that from healthy controls. Among these, higher levels of *Actinobacteria*, *Fusobacteria,* and *Acidobacteria* were seen in samples of patients with SCRC compared to that of those patients with HCRC (Figure 3A). Through LEfSe analysis, significantly different taxa were identified at several levels between samples of patients with HCRC and that of those with SCRC (Figure 3B). We then focused on the most prominently different genera, which was highly abundant in the samples of cases with SCRC contrast that of HCRC. Throughout using partitions all over the medoids clustering algorithm on the 111 different genera abundance profiles, results showed that the microbiota communities were converted into two clusters (Figures 3C, 3D). Among the top 20 highly abundant genera, *Parvimonas* was particularly prominent in the feces specimens of patients with SCRC, whereas the other 19 genera were found to be more in the cases of patients with HCRC (Figure 3E). Consistent with these findings, a higher abundance of *Parvimonas* was noted in the samples obtained from patients with SCRC in relation to samples obtained from healthy controls (Figure 3F), indicating that *Parvimonas* possibly involves in CRC progression.

Using Spearman's correlation coefficient analysis, we further evaluated the correlation of the top 30 genera that were highly abundant in samples obtained from patients with SCRC and HCRC. *Parvimonas* was positively correlated with *Brevibacillus, Methylobacterium, Granulicella,* and *Phenylobacterium*, all of which were more enriched in SCRC conditions compared to HCRC conditions (Figure 3G). Among the genera most abundant in SCRC, *Parvimonas* and *Fusobacterium* were found to be positively correlated (Figure 3H).

Verification of *Parvimonas* and *Fusobacterium* using targeted qPCR

We measured the abundance of *Parvimonas* and *Fusobacterium* using qPCR in all subjects (10 HNPCC patients, 14 SCRC, and 14 controls). As for *Parvimonas,* the abundance in SCRC was higher than in HCRC samples ($p < 0.001$), which is consistent with the results of 16S rDNA V4 gene sequencing. Meanwhile, there were also significantly differences between SCRC samples and controls ($p < 0.001$), without differences between HCRC and healthy subjects ($p = 0.348$) (Figure 4A). Interestingly, the abundance of *Fusobacterium* in SCRC group and HCRC group was both higher than healthy individuals ($p = 0.001$, $p < 0.001$), whereas qPCR results revealed that *Fusobacterium* was not more enriched in SCRC samples compared to HCRC ($p = 0.280$) (Figure 4B).

DISCUSSION

Accumulating studies have illustrated the structural differences of the gut microbiome between CRC and healthy subjects. Several kinds of bacteria and the mechanism of promoting tumor toxicity have been shown to be associated with CRC pathogenesis. Independent studies[14-17] have shown that the feces and tumor biopsy cases of CRC were added with *Fusobacterium nucleatum* compared with that of the controls, and subsequent studies have indicated that *F. nucleatum* promoted CRC by modulating immunity (increasing inhibitory receptors of natural killer cells[18] and myeloid-derived suppressor cells[14]), virulence factors (FadA and Fap2)[19-22], microRNAs (miR-21)[23], as well as bacterial metabolism[14,24]. *Escherichia coli* has also been identified to be abundant in CRC tissues[22,25-28]. Strains contain a pathogenicity island (54kb polyketide synthases) that synthesizes a peptide-polyketide hybrid genotoxin, which could induce DNA damage[29]. *Bacteroides fragilis* potentiates intestinal tumorigenesis through binding to epithelial receptor of colon, and then activates NF-κB and Wnt signaling pathways[30-32]. Despite biological mechanisms potentially contributing to colorectal tumorigenesis, this association cannot only be inferred from cross-sectional human studies comparing CRC and healthy individuals. Evidence suggesting gut microbiota as a causative factor of CRC was studied in $APC^{min/+}$ mice, a genetic model of CRC, where free mice promoted 2-fold and small

intestine and its tumors are less. However, no significant differences were noted in the rest of the intestinal tract, including the colon, indicating that microbial state has no significant effect in $APC^{min/+}$ mice[33].

As we know, it is the first experiment which characterizes the microbiome dissimilarity between patients with SCRC and HCRC. We have presented significant differences in the microbiota configurations between two groups. With the phenotypes of CRC being quite similar, the marked variations in the fecal microbiome cannot be justified as a consequence of CRC. If microbial dysbiosis occurs as a consequence of CRC, microbiota configurations of SCRC and HCRC would be comparable. Therefore, this finding could serve as evidence supporting the theory that gut microbiota causes the promotion of CRC. Significant over-representations of *Parvimonas*, a specific genus associated with CRC, were also observed in the fecal samples of SCRC contrast to HCRC. This suggests that *Parvimonas* may be a bacterial genus specific to CRC. A recent review based on studies conducted on CRC dysbiosis published within the last decade consistently detected *Parvimonas, Fusobacterium,* and *Streptococcus* in both CRC tissue biopsies and fecal samples, compared to those of healthy controls[34]. The *Parvimonas* genus contains a single gram-positive species, *Parvimonas micra*, which is an anaerobic coccus causing oral infections like *F. nucleatum*[35,36]. Carretero et al.[37] reported bacteraemia due to *P. micra* in a patient with esophageal carcinoma. A meta-genomic study showed a significant enrichment of *P. micra* across five different CRC cohorts and an intense co-occurrence pattern of *F*. *nucleatum* and *P. micra* [38]. Horiuchi et al.[35] revealed that *F. nucleatum* and *P. micra* existed synergistic effects in causing apical periodontitis lesions. Similarly, this study indicated a positive correlation between *Parvimonas* and *Fusobacterium*. However, the exact role of *Parvimonas* in CRC is not investigated in our study, which is the main study limitation and required further studies in the future.

In conclusion, difference in gut microbial composition among cases of SCRC and HCRC suggests that (i) gut microbiota may have diverse impacts on HCRC and SCRC; (ii) fecal microbial dysbiosis could be used to ascertain a causative role in the development of SCRC; and (iii) *Parvimonas*, due to strong co-occurrence with *Fusobacterium*, may be one of causal bacterial candidates in CRC-associated dysbiosis. Further meta-genomic sequencing on the microbiome data of patients with SCRC and HCRC are warranted and may eventually advance the perception the impact of microbiota in its pathogenesis of CRC and define the underlying mechanism.

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Disclosure of conflict of interest

None.

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TABLES

Table 1. **Characteristics of patients with HCRC†**

Code	Gender	Age of diagnosis	Site	‡MMR mutation sites and mutation type
D14091172	male	29	rectum	hMSH2 exon-13 IVS13-2 A>C
D14091168	male	38	right colon	hMLH1 exon-19 c.2141G>A
D14091166	female	28	right colon	hMLH1 exon-19 c.2141G>A
D14091182	male	49	right colon	hMSH2 exon-8 c.655 A>G
D14091174	male	43	right colon	hMLH1 exon-6 c.503_4 insA
D14091170	male	51	left colon	PMS1 exon-5 c.402 G>C
D14091176	female	61	left colon	hMLH1 exon-11 c.910 T>A
D14091184	male	47	left colon	hMLH1 exon-16 c.1823 C>A
D14091187	female	28	left colon	hMLH1 exon-2 c.199dupG
D14091178	female	47	right colon	hMSH2 exon-10 c.1571 G>T

†HCRC, hereditary colorectal cancer; ‡MMR, mismatch repair

Table 2. **Characteristics of patients with SCRC†**

Code	Gender	Age of diagnosis	Site
D14091154	male	36	left colon
D170611269	male	56	left colon
D170611270	male	65	left colon
D170611271	female	55	right colon
D170611272	male	55	left colon
D170611273	male	65	left colon
D170611274	female	39	rectum
D170611275	female	38	right colon
D170611276	female	56	rectum
D16055167	female	43	left colon
D16055164	male	39	left colon
D16073216	male	51	left colon
D16055165	female	36	rectum
D16055166	male	47	left colon

†SCRC, sporadic colorectal cancer

FIGURE LEGENDS

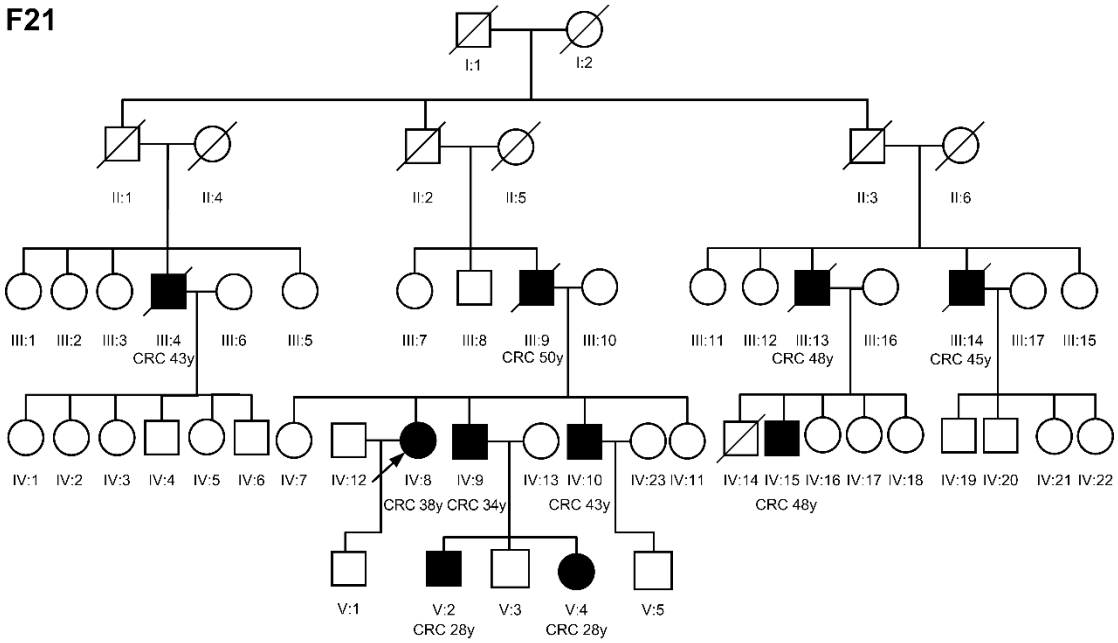

Figure 1. **Pedigree of the family 21. Affected family members with CRC have been represented with black symbols, along with the age at diagnosis. The proband has been indicated with arrows.**

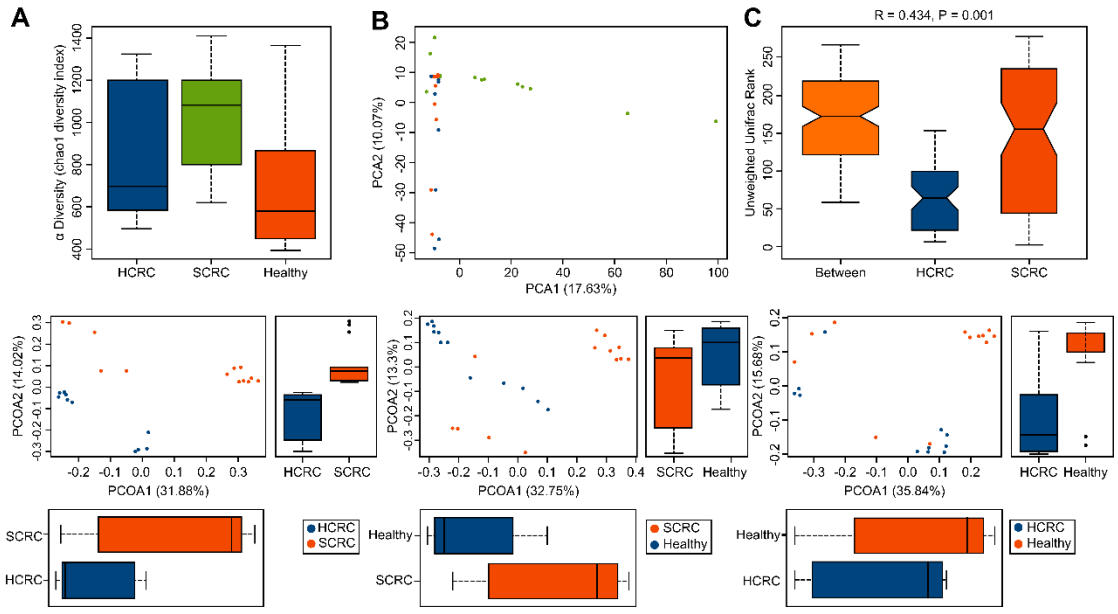

Figure 2. **The difference in the fecal microbiota among patients with SCRC, HNPCC (referred to as HCRC), and healthy controls.**

(A) alpha diversity (chao1 index). SCRC & HCRC p = 0.011, SCRC & HCRC p = 0.206, HCRC & Healthy p = 0.189. (B) SCRC and control groups differ due to the fact displayed by PCA scores' plot based on relative abundance of OTUs compared with the HCRC group. (C) Based on Anosim analysis and PCoA, the inset shows microbiota community structure from people suffering from SCRC differs significantly compared with patients with HCRC (p = 0.001), and significant differences also can be identified between patients with CRC and healthy controls (SCRC and Healthy, p = 0.002; HCRC and Healthy, p = 0.031).

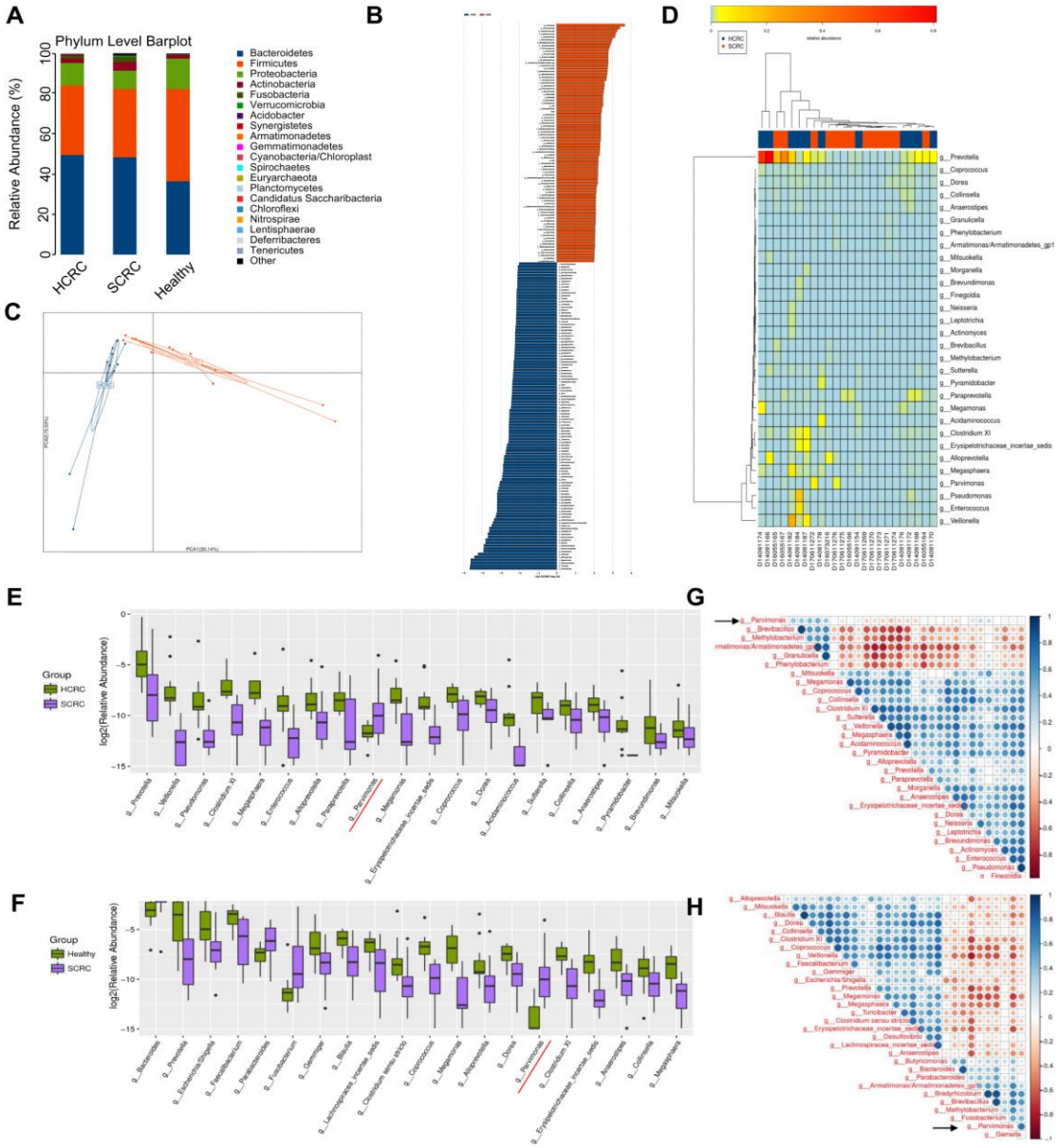

Figure 3. **Differential microbiota taxa between the SCRC and HCRC groups.**

(A) Differentially enriched microbiota phylum in the samples obtained from patients with SCRC included *Bacteroidetes, Actinobacteria*, *Fusobacteria,* and *Acidobacteria* compared with that in the fecal samples of patients with HCRC. (B) LEfSe analysis revealed that a greater number of taxa, from class to genus levels, were found to be abundant in specimens obtained from patients with SCRC than those obtained from patients with HCRC. (C) PCA and (D) Heatmap of the presence of differential genera showed significantly different genus communities between the fecal specimens obtained from patients with SCRC and HCRC. (E) Differentially enriched microbe genera between samples obtained from patients with HCRC and those with SCRC were impacted by FDR statistics. Deseq analysis with the help of it was carried out (only after correction, $P < 0.05$). Besides, the 20 most abundant have been shown). (F) Variant genera between SCRC and healthy patient samples ($p < 0.05$ and the 20 most abundant have been shown). (G) Correlations of the 30 most abundant genera in samples obtained from SCRC and HCRC patients. It is necessary to calculate the correlation coefficient of Spearman. At the same time, it should be determined that all paired comparisons are statistically significant. When significantly correlated. The blue circle indicates that their

relationship is in a positive proportion, and the red circle indicates the opposite result. The size and shadow area directly reflects the high or low degree of correlation. That is, deep shadows and large dimensions suggest a deeper correlation. (H) Spearman's correlation coefficient on dominating genera in SCRC compared with healthy controls showed that there were strong co-occurrence patterns between *Parvimonas* and *Fusobacterium.*

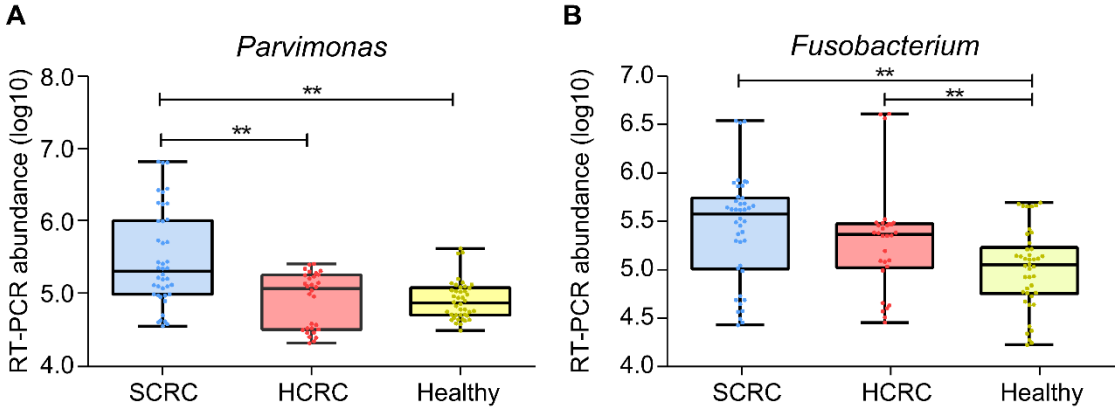

Figure 4. **Quantitative PCR (qPCR) results confirmed the analysis of 16S rDNA V4 gene sequencing.**

(A) *Parvimonas* (B) *Fusobacterium.* The abundance of the both two genera in SCRC samples were significantly higher than in healthy subjects (p < 0.001, p =0.001). Interestingly, in HCRC samples, that situation was inconsistent for two genera. *Parvimonas* was more enriched in SCRC faece samples than in HCRC (p < 0.001), and there were not differences between HCRC and Healthy samples (p = 0.348). Whereas the abundance of *Fusobacterium* did not differ between SCRC and HCRC subjects (p = 0.280), both higher than the healthy (p = 0.001, p < 0.001).